Supporting Information

Briscoe et al. 10.1073/pnas.0910085107

SI Materials and Methods

PCR, Cloning, and Sequencing of *H. erato* UV Opsins. *H. erato* cDNA was synthesized from total RNA extracted from a single adult head (TRIzol: Gibco-BRL) using a Marathon cDNA amplification kit (BD Biosciences, Clontech). Rapid amplification of cDNA ends (3′ and 5′ RACE) products were amplified by degenerate and gene-specific primers, paired with the kit's adaptor primer, using BD Advantage Polymerase (BD Biosciences) and the following touchdown PCR protocol: 1 min at 95 °C; 5 cycles of 30 s at 95 °C, 1.5 min at 68 °C; 5 cycles of 30 s at 95 °C, 30 s at 65 °C, and 1.5 min at 68 °C; 5 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1.5 min at 68 °C; 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1.5 min at 68 °C, and 10 min at 68 °C. PCR products were cloned into pGEM-T Easy vector (Promega) and screened for inserts with EcoRI (New England Biolabs). A total of 220 plasmids were screened, 24 of which were initially sequenced with Big Dye 3.1 sequencing kit (Applied Biosystems) at the DNA core facility in University of California, Irvine. To ensure that all opsins were recovered, the remaining plasmids were screened by multiplex PCR using several pairs of gene-specific primers mixed in the same PCR. A total of 105 clones of the appropriate size that did not amplify any PCR product were sequenced.

Identification of UV Opsin cDNAs from Other Butterfly Species. One-step reverse transcriptase PCR (RT-PCR) was performed to amplify *UVRh* opsins from total RNAs for the other eight species in the genus using AffinityScript multiple temperature cDNA synthesis kit (Stratagene) and the species-specific primers. Primers for *Heliconius charithonia*, *H. doris*, and *H. hortense* were able to amplify both *UVRh1* and *UVRh2* opsins in the same PCR; we cloned the PCR products into pGEM-T easy vector and sequenced eight clones of *H. charithonia*, 40 clones of *H. doris*, and 14 clones of *H. hortense* to isolate different UV opsins.

UV opsins of five basal heliconiines (*Agraulis vanillae*, *Dione moneta*, *Dryas iulia*, *Eueides vibilia*, and *Speyeria mormonia*) were obtained using the same method as used for *H. erato*.

Phylogenetic Analysis. GenBank accession nos. of the nymphalid UV opsins used in reconstructing the gene tree shown in Fig. 1 (besides the ones shown in Table S1) are as follows: *Danaus plexippus* (AY605546), *Euphydryas chalcedona* (EU449014), *Limenitis archippus* (EU449016), *Limenitis arthemis* (AY918901), *Nymphalis antiopa* (AY918892), and *Vanessa cardui* (AF414074).

Epi-Microspectrophotometry of *Dryas iulia*. We used an epi-microspectrophotometer (MSP) to measure eyeshine reflectance spectra, photoconvert the LW rhodopsin to its metarhodopsin photoproduct, and monitor the dark-processes of metarhodopsin decay and rhodopsin recovery. Difference spectra were analyzed by nonlinear least-squares regression to Bernard's 1987 polynomial templates (1) for both rhodopsin and metarhodopsin, to estimate the absorbance spectrum of the long wavelength (LW) rhodopsin and its wavelength for maximal absorbance λ_{max}.

The experiment for estimating R555 of *D. iulia* began with MSP setup on the dorsal pole of an eye using 670 nm light, dark adapting for 150 min, and then measuring the eyeshine reflectance spectrum. The eye was then partially bleached of LW rhodopsin by delivering a 22-min series of intense 630 nm 2-s flashes every 30 s. The eyeshine reflectance spectrum was measured 7 min later.

Estimation of R470 for *D. iulia* began with MSP setup on a latero-equatorial region on the other eye of the same individual. After setup with 670 nm light the eye was treated with 20 min of intense 620 nm 2-s/30-s flashes. After resting in the dark for 30 min the eye was treated with 5 min of intense 450 nm 2-s/30-s flashes. A difference spectrum was computed from reflectance spectra measured before and 6 min after the blue flashes.

Estimation of R385 for *D. iulia* became possible after determination of R555 and R470. During the experiments on the dorsal pole mentioned above, the eye was dark adapted for 264 min after which a photoproduct-free reflectance spectrum was measured. We analyzed that reflectance spectrum using the procedure described for *H. erato* (see main text). Stripping 1.7 optical density (OD) of R555 and 0.6 OD of R470 left a residual that was very well fit by 1.2 OD of an R385 template. Least-squares fit to the normalized absorbance spectrum was 384.5 nm ± 1.0 nm, 95% confidence bounds = 382.5 nm to 386.6 nm. SD of the fit was 0.04 nm.

Computational Analysis of Electrophysiological Data. G. Struwe (2, 3) published early electrophysiological spectral sensitivity functions for photoreceptor cells of heliconiine butterflies using both intracellular and ERG techniques. His work on *H. numata* is particularly useful because he presents intracellular data for three spectrally distinct types of cell. Although these are not clean, single-unit recordings and contain systematic errors, they are nonetheless very useful because one is dominated by red sensitivity (Fig. S1*A*, squares), another by blue sensitivity (Fig. S1*B*, squares), and another by UV sensitivity (Fig. S1*C*, squares). We show that analysis using these data plus his ERG data (Fig. S1*D*, squares) produces tight estimates for the λ_{max}'s of four visual pigments. The same receptor system must drive all four measurements, in different ways. The challenge is to determine their spectral positions and relative contributions.

Intracellular Sensitivity Functions. We created a computational model in which recordings are driven by linear, weighted sums of responses from four spectral types of receptor, each of which is represented using Bernard's eighth-order polynomial rhodopsin template (1) of λ_{max} values L1, L2, L3, and L4 and weighting coefficients W1, W2, W3, and W4, respectively. Various versions of this model are fitted to Struwe's data via nonlinear least squares regression.*

If all eight parameters are allowed to be free, results are quite poor with huge standard errors of wavelength estimates and large standard deviation (SD) of regression. The only bright spot is an estimate of 554 ± 4 nm for L4 of the red-dominated data. This estimate for the λ_{max} of the long wavelength visual pigment is similar to those we obtained previously (550–560 nm) for several other *Heliconius* species using epi-microspectrophotometry (4). We do much better under the reasonable assumption that the two UV rhodopsins of *H. numata* are the same as for *H. erato*, R355

*Our computational model assumes a normal distribution of errors in the data, independent of wavelength and experimental conditions. Three situations in Struwe's data violate that assumption. First, is a systematic error in quantum measurement of the UV points at 310 nm (ERG data) and 330 nm (intracellular data) shown in Struwe's Figs. 1 and 2 (2,3). Radiometry of short wavelength UV light is technically difficult. Sensors typically used for these measurements are more sensitive to visible and infra-red light than to UV light, so keeping stray light out of short wavelength UV measurements is difficult but very important. Data points at 310 nm and 330 nm are low by 0.2–0.3 log units compared to expected values based on full analysis of remaining data. We exclude those data from Fig. S1 *A*, *B*, *C*, and *D*. Second, the UV-dominated intracellular data shown in Struwe's original figure are really strange for wavelengths beyond 470 nm, suddenly jumping from a trough at 450 nm to a plateau between 470 nm and 510 nm. These data cannot be explained by linear combinations of known receptors. We exclude those data from Fig. S1*C*, but retain data between 350 and 450 nm to help evaluate our estimates of L1 and L2. Third, the fifth receptor type, a laterally filtered red receptor, produced by red filter pigments in combination with a long wavelength visual pigment, characterized in *H. erato*, contributes to the ERG for wavelengths 590 nm and greater. We exclude those data from Fig. S1*D*.

and R398, and then fix L1 and L2 at those values. Suppose we seek L3 from the blue-dominated data. Fix L1 = 355, L2 = 398, and also L4 = 554 nm, based on results stated above. The fit now has only five free parameters, producing a standard deviation of regression of SD = 0.041 and an estimate L3 = 467 ± 2 nm with 95% confidence limits of 463–472 nm. We choose 465 nm.

Similarly, if we fix L1 = 355, L3 = 465, L4 = 550, and allow L2 to be free, the fit to the blue-dominated data are: SD = 0.027 and estimate of L2 = 399 ± 3 nm with 95% limits 393–406 nm. This is consistent with our choice of R398. Or, if we fix L2 = 398, L3 = 465, L4 = 550, and allow L1 to be free, results are: SD = 0.027, L1 = 349 ± 6 nm with 95% limits 335–364 nm, consistent with our choice of 355 nm.

If one considers the UV-dominated data between limits 350 nm and 450 nm, it is well fit by SD = 0.023 with L1 = 355, L2 = 398, and L3 = 465. If one considers the competing hypothesis of only a single UV visual pigment, that model performs poorly having SD = 0.101 with Luv = 384 ± 4 nm, with an oscillating fit (Fig. S1*C*, thin line). Similarly if one returns to the two other intracellular data sets and forces a single-UV fit, results are also poor: SD increasing from 0.041 to 0.052 for the red-dominated data (Fig. S1*A*, thin line) and from 0.026 to 0.047 for the blue-dominated data (Fig. S1*B*, thin line).

ERG Sensitivity Function. From the three intracellular recordings we learned that there are four visual pigments with λ_{max} = 355 nm, 398 nm, 465 nm, and 550 nm. Furthermore, the hypothesis of a single UV visual pigment is not supported. Suppose the four λ_{max}'s are fixed at those values: how tightly do the fitted curves fit the electroretinogram (ERG) data? There are then 13 data points and only 4 free parameters, so it is a pretty good test. Results are excellent, with SD = 0.056 (Fig. S1*D*, thick line). If one insists on only one UV visual pigment of λ_{max} to be determined, but keep R465 and R550 fixed results are SD = 0.080 and R385 ± 4 and the fitted curve is poor (Fig. S1*D*, thin line).

In summary, there are two UV visual pigments in *H. numata* that are the same as found in *H. erato*: R355 and R398. The competing hypothesis of only a single UV visual pigment is not supported. The other two visual pigments of *H. numata* are R465 and R550.

Characterizing the Wing Reflectance Spectra in the UV Range. Wing reflectance spectra from 11 non-*Heliconius* heliconiine species and 9 *Heliconius* species were examined in the present study (see Table S1 and Fig. S2 for details). Total numbers of specimens for each species (and one subspecies) included in our spectral characterization are listed in Table S1. The wing areas for which the reflectance spectra were taken vary across species, but generally any distinct color patch on the wing (both dorsal and ventral sides of forewing and hindwing) that is within our probe size range of the spectrometer (1–2 mm in diameter) was sampled (9–14 patches per specimen). We note that the silver patches of *S. mormonia*, *A. vanillae*, and *D. moneta* as well as presumably the blue of *H. doris* are structural colors and that reflectance spectra of structural colors are angle dependent. We measured these wing patches using our 45° fixed angle probe holder (Fig. S2), but excluded them from further analysis due to the need for more exhaustive sampling over a range of angles. It is unlikely that this will have affected our all colors results (see below) because silver patches function like white-light mirrors, reflecting light over a broad typically flat range.

To minimize individual specimen variation, reflectance spectra measured from the same color patch of the wing were averaged (3–14 specimens per species, except *Eueides procula* and *E. heliconioides*). Average wing reflectance spectra of the sampled areas for all species are shown in Fig. S2. Pairwise correlation analysis among spectral measurements of five randomly selected *H. erato* specimens showed that the correlation coefficients range from 0.86 to 0.99 in each sampled wing area. This suggests that these wing measurements are generally consistent across different specimens within the same species, thus the average spectrum is a reasonable measure of the wing reflectance in our spectral characterization. In addition, we also tested the reliability of repeated measurements by comparing the reflectance spectra of the same wing area taken two weeks apart from the same specimen. The correlation coefficients between two repeated measurements of the same wing area from five specimens of *H. erato* ranged from 0.94 to 0.99 on average, which suggests that reflectance of the wing pigments is stable over time, and our spectral measurements are consistent across different repeats.

Collecting Environmental Light Data. We visited two sites in the Mixe region of Oaxaca, Mexico (Choapam, 760 m and Amaltepec, 1,600 m) in October 2009 where we first caught a number of heliconiines with nets (including *Dryas iulia*, *Dione moneta*, *Eueides aliphera*, *E. lineata*, *Heliconius charithonia*, *H. hortense*, *H. ismenius*, and *H. hecalesia*). Importantly, although we did not catch them on this trip, *Agraulis vanillae*, *E. isabella*, and *H. erato* had also been collected at the Choapam site in the proceeding months (July–September 2009) and the material deposited in the Museo de Zoología, Mexico City as part of long-term faunistic studies. We then took irradiance measurements at localities within these sites where the butterflies had been caught or were observed to fly using an Ocean Optics USB2000 spectrometer and either a CC-3 cosine-corrected 100 μm or 400 μm fiber. The localities and weather conditions where the butterflies were caught can be roughly classified into forest shade and cloudy conditions (*H. hortense*) and open habitat and sunny conditions (all other species). For the cloudy forest conditions, irradiance measurements were taken by holding the fiber parallel to the ground (sideways) and for the sunny open conditions irradiance measurements were taken by holding the fiber perpendicular to the ground (facing up). Integration times suitable for the dynamic range of the scope were determined by keeping the raw measurements between 3,000 and 4,000 counts. Individual replicates were averaged (n = 3–8). Data were transformed together with calibrated lamp measurements using the LS-1-CAL calibrated light source into units of photon/cm^2/nm/s, smoothed in MatLab using a Gaussian function (3 nm bandwidth) and then normalized for color space modeling (see below).

Color Space Modeling. To understand the possible signal content of the *Heliconius* yellows compared to non-*Heliconius* yellows, we compared their discriminability through the eyes of *H. erato* by modeling the color space of all possible classes of trichromatic ommatidia of *H. erato* and *D. iulia*. The color space models of Vorobyev and Osorio (5) as implemented in the program SPEC written by Jarrod Hadfield (see equations below) were used to estimate the discriminability (ΔS) of pairs of wing reflectance spectra. This model has been extensively used to model vertebrate visual systems and that of honeybees (6).

We first calculated von Kries' transformed quantum catch, q_i, for each photoreceptor type to account for color constancy, which lepidopterans have been shown to have (7), as:

$$q_i = \frac{\int_\lambda R_i(\lambda)S(\lambda)I(\lambda)d(\lambda)}{\int_\lambda R_i(\lambda)I(\lambda)d(\lambda)}, \qquad \text{[S1]}$$

where $R_i(\lambda)$ = sensitivity of the receptor type i, $S(\lambda)$ = the reflectance spectrum of the wing color, $I(\lambda)$ = the irradiance spectrum, and $d(\lambda)$ = 5 nm from the interval of 310–695 nm. Spectral sensitivity curves, $R_i(\lambda)$ for *H. erato* and *D. iulia* were generated using the rhodopsin template of Bernard (1) based on λ_{max} values for the photoreceptors found in the eyes of each species described in the main text. For *H. erato* λ_{max} values of the four visual pigments corresponded to 555 nm (long wavelength, LW), 470 nm (blue) 398 nm and 355 nm (UV). For *Dryas iulia,* λ_{max} values of the three visual pigments corresponded to 555 nm (long wavelength), 470 nm (blue) and 385 nm (UV), respectively (Fig. 2). Both open habitat and forest shade irradiance spectra

shown in Fig. S5 were used in the calculations along with the parameters specified below.

We next calculated the signal f of receptor i as

$$f_i = \log(q_i). \quad \text{[S2]}$$

The difference in receptor signals (Δf_i) for two stimuli, a and b was calculated as

$$\Delta f_i = f_{i,a} - f_{i,b}. \quad \text{[S3]}$$

Finally, to compare the discriminability (ΔS) of two stimuli: for a trichromat,

$$(\Delta S)^2 = \frac{e_1^2(\Delta f_3 - \Delta f_2)^2 + e_2^2(\Delta f_3 - \Delta f_1)^2 + e_3^2(\Delta f_1 - \Delta f_2)^2}{(e_1e_2)^2 + (e_1e_3)^2 + (e_2e_3)^2} \quad \text{[S4]}$$

where e_i represents noise in receptor i and is calculated as follows:

$$e_i = \sqrt{\frac{\left(1/\left(\log\left(T^{\frac{(Q_{i,a}+Q_{i,b})}{2}}\right)\right)^2 + w_i^2\right)}{n_i}}. \quad \text{[S5]}$$

The Weber fraction, w_i, is the inherent noise-to-signal ratio in receptor cells of type i, independent of quantum catch, and n_i is the relative abundance of receptor type i in the retina, and T is a scaling function, which relates Q_i (a proportion of the maximal receptor catch) to an absolute quantum catch value. We used a value of $w_i = 0.05$ as has been used in other models of butterfly color vision (8). The relative abundance of receptor types in either the *H. erato* or *D. iulia* eye used for species recognition, n_i, is unknown. Recent (8) as well as older studies have found that butterflies use a subset of photoreceptor cells for specific tasks rather than all possible combinations found in their retina. The most conservative assumption we could make based on available in situ hybridization data from the *H. erato* retina (9, 10) is that the individual ommatidium is the simplest unit for color vision because each photoreceptor cell in an ommatidium shares a single lens. Based on the in situ data, in which either *UVRh* or *BRh* are expressed in the R1 and R2 photoreceptors cells and *LWRh* is expressed in the R3–R8 photoreceptor cells (see main text) we decided to model all possible classes of trichromatic ommatidia in the *Heliconius* and *Dryas* eye. For *Heliconius*, we modeled the three possible subtypes of trichromatic ommatidia with the following photoreceptor ratios: 1 UV + 1 V + 6 LW, 1 UV + 1 B + 6 LW, and 1 V + 1 B + 6 LW, where UV represents the 355-nm rhodopsin and V represents the 398-nm rhodopsin. For *Dryas*, we modeled one type of trichromatic ommatidium: 1 UV + 1 B + 6 LW. We note that we do not yet know whether inputs from each of these three receptor classes are in fact being compared in the context of species recognition and that results of the model are highly sensitive to the relative abundance of the receptor classes used in the calculations. Also we ignored in our model the possibility that both *H. erato* and *D. iulia* may have an additional long wavelength photoreceptor class in the eye due to the presence of a heterogeneously expressed red filter pigment (*Introduction*) and considered only the hypothetical comparison of a trichromatic color vision system. This is because we currently do not have any anatomical data for the relationship between the red filter pigment and opsin expression, nor do we have physiological recordings that would give us the shape of the spectral sensitivity curve of individual photoreceptor cells subject to the effects of the red filter. Lastly, we used $T = 10{,}000$ corresponding to bright illumination and $T = 500$ corresponding to dim illumination.

To understand the possible functional advantage of the *Heliconius* yellow compared to the non-*Heliconius* yellow, we divided all measured yellow reflectance spectra into two categories, *Heliconius* yellow and non-*Heliconius* yellow and calculated all pairwise ΔS within each of these categories under sunny open habitat and forest shade illumination conditions (similar to Endler 1993 daylight and forest shade spectra) (11) for all possible classes of trichromatic ommatidia in the *Heliconius* and *Dryas* eyes. The ΔS values represent the Euclidean distance separating two colors in *Heliconius* or *Dryas* color space and larger numbers represent more easily distinguished colors. Under the original Vorobyev-Osorio model (1998) (5), the threshold criterion of $\Delta S = 1$ was defined as a just noticeable difference between a pair of compared colors. Due to the difficulty in determining the true noise values of the butterfly visual system, and the potential impact of receptor noise values on model predictions (12), we have chosen ΔS threshold values of one (original threshold), two, and three for comparison similar to threshold values used in other studies (13). We found that the percentage of pairs of *Heliconius* yellows exceeding the thresholds of one, two, and three JNDs was much higher than for non-*Heliconius* yellows whether illuminated in forest shade or open habitat or under dim or bright illumination, and irrespective of whether they were viewed through the eyes of *Dryas* or *Heliconius* (Fig. 4 and Table S3). This result indicates that by evolving a new mechanism for producing yellow colors, *Heliconius* has significantly increased the number of distinct yellow colors on the wing compared to non-*Heliconius* species. We also found under all studied illuminant conditions except one, *Heliconius* may have a slight advantage over *Dryas* especially in discriminating *Heliconius* from non-*Heliconius* yellows, if they in fact compare signals from photoreceptor cells expressing UVRh1, UVRh2, and LWRh (Fig. S6 and Table S3). As we noted in the main text, it is equally plausible that other photoreceptor combinations are used for species recognition, in which case the *Dryas* visual system may have an advantage (Table S3). Therefore, we note that as with any theoretical work, it will be of utmost importance to validate these predictions through future behavioral testing.

Correlated Trait Analysis. To characterize a species' wing pigments in the UV range for the correlated trait analysis of all colors (see main text for coding of UV-yellow pigment), we computed the maximum slope and maximum reflectance of the average spectrum within the range of 310–390 nm measured from each wing area. The maximal slope was determined by seeking the largest absolute value (i.e., regardless of whether it was positive or negative) of the first derivative of the reflectance spectrum at 1-nm increments. A species was arbitrarily coded as having spectral variation in the UV range (code = 1) if it met two criteria: (*i*) the absolute value of the maximal slope of at least one wing reflectance spectrum exceeded 0.1, and (*ii*) the maximal percentage of reflectance of this spectrum was at least 15% or more in the UV range. If either of these criteria was not met then the species was considered to have little spectral variation in the UV and coded as 0. This procedure ensures that the strength of UV signals is significantly high enough for visual perception. We also classified the species according to two other criteria corresponding to increasing spectral variation in the UV and increasing maximum reflectance in the UV (Fig. S2 legend). For the visual pigment trait, a species with two UV opsins was coded as 1 and a species with 1 UV opsin was coded as 0. The results of the correlated trait analysis are independent of the choice of these criteria ($P = 0.004$–0.01). Although under all three criteria, spectral variation (i.e., coloration) in the UV was more likely to occur on the wings of butterflies in the presence of two UV opsins in the eyes than in the presence of only one, we note that in general correlation does not imply causation.

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Table S1. Heliconiine specimens used in this study

Tribe	Species	Sex	ID no.	Collecting locality of eye tissue	GenBank accession no.	No. specimens measured for wing reflectances
Argynnini	*Speyeria mormonia*	Male	SMOR18	Tioga Pass, Mono County, CA	EU449019	3
Heliconiini	*Agraulis vanillae*		AVAN	Huntington Beach, CA	EU449018	7
Heliconiini	*A. v. lucina*					3
Heliconiini	*Dione moneta*		AB55	El Guajolote, Loxicha, Oaxaca, Mexico	**GQ451891**	7
Heliconiini	*Dryas iulia*	Male	DRJ1	Texas	**GQ451890**	4
Heliconiini	*Eueides vibilia*	Male	R83	Near Yurimaguas, Peru	**GQ451892**	4
Heliconiini	*E. heliconioides*					2
Heliconiini	*E. isabella*					3
Heliconiini	*E. procula*					1*
Heliconiini	*E. surdus*					3
Heliconiini	*E. thales*					3
Heliconiini	*H. charithonia*	Female Male	HCH11 HCH29	N/A Chilpancingo, Guerrero, Mexico	**GQ451893** (1) **GQ451894** (2)	4
Heliconiini	*H. cydno*		HCY10	Atlantic slope, Costa Rica	**GQ451895** (1) **GQ451896** (2)	4
Heliconiini	*H. doris*	Male	R31	La Merced, Peru	**GQ451897** (1) **GQ451898** (2)	4
Heliconiini	*H. erato*	Female	HER1	Sirena, Costa Rica	AY918904 (1) **AY918905** (2)	14
Heliconiini	*H. hortense*		AB36	Pluma Hidalgo, Finca Aurora-San Isidro, Oaxaca, Mexico	**GQ451899** (1) **GQ451900** (2)	5
Heliconiini	*H. melpomene*	Male	HMP12	El Rodeo, Costa Rica	**GQ451901** (1) **GQ451902** (2)	3
Heliconiini	*H. numata*	Male	R78	Near Yurimaguas, Peru	**GQ451903** (1) **GQ451904** (2)	3
Heliconiini	*H. pachinus*	Female	HPA1	Sirena, Costa Rica	**GQ451905** (1) **GQ451906** (2)	4
Heliconiini	*H. sapho*		HSP11	Atlantic slope, Costa Rica	**GQ451907** (1) **GQ451908** (2)	3
Heliconiini	*Podotricha telesiphe*					3*

New sequences are indicated in bold.
*Reflectances were taken from pinned museum specimens.

Table S2. Log likelihood values and parameter estimates for *UVRh2* branch-sites test of selection

Model	λ	Site class	Proportion	Background ω	Foreground ω	Positively selected substitutions
A	−7375.33	0	0.745	0.030	0.030	
		1	0.052	1.000	1.000	
		2a	0.189	0.030	9.625	D35E, A37E, L60M*, F77Y, T121I, A123S/T, **M132V, A145L,** M171L, A179T*,
		2b	0.013	1.000	9.625	M185L, **N189R,** V190I, S202A*, T204S, **V218T,** C224F, S225T/A*, V/L227A, F228I*, M230L, F232L, I234M, F236Y, F289Y*, T312S, V321I, A361V
Null	−7381.31	0	0.314	0.030	0.030	
		1	0.022	1.000	1.000	
		2a	0.621	0.030	1.000	
		2b	0.044	1.000	1.000	

BEB-identified positively selected sites are inferred at $P = 95\%$ with those reaching 99% shown in bold. LRT = $2\Delta\ell = 12.0$, df = 1, $P = 0.0005$.
*Homologous to human red cone pigment amino acid residues 59, 180, 202, 227, 230, and 277.

Table S3. Percentage of pairs of *Heliconius* and non-*Heliconius* yellows compared that exceed thresholds of one, two, and three just noticeable differences (JNDs) as modeled through all possible subtypes of trichromatic ommatidia in the *Heliconius* or *Dryas* eye under dim or bright illumination in forest shade or open habitat

				JND 1			JND 2			JND 3		
Light	Habitat	Color	Subtype	%	Lower CI	Upper CI	%	Lower CI	Upper CI	%	Lower CI	Upper CI
Dim	Forest	Non-HelY	UV + V*	**54.2**	47.8	60.4	**15.0**	10.9	20.0	**2.4**	0.9	5.1
			UV + B	37.2	31.2	43.4	6.3	3.7	10.1	0.4	0.0	2.2
			V + B	49.8	43.5	56.1	11.5	7.8	16.1	0.4	0.0	2.2
			*Dryas**	47.8	41.5	54.2	10.3	6.8	14.7	0.4	0.0	2.2
		HelY	UV + V*	**72.6**	67.7	77.3	**47.6**	42.3	53.0	**24.2**	19.8	29.1
			UV + B	65.5	60.3	70.5	29.6	24.9	34.7	14.8	11.3	19.0
			V + B	67.0	61.8	71.9	34.2	29.2	39.4	19.9	15.9	24.5
			*Dryas**	68.9	63.8	73.8	37.6	32.5	42.9	20.5	16.4	25.1
	Open	Non-HelY	UV + V*	**70.0**	63.9	75.5	**34.4**	28.6	40.6	**13.0**	9.2	17.8
			UV + B	54.2	47.8	60.4	21.7	16.8	27.3	6.3	3.7	10.1
			V + B	62.1	55.8	68.1	26.5	21.2	32.4	6.3	3.7	10.1
			*Dryas**	61.3	55.0	67.3	28.5	23.0	34.5	5.9	3.4	9.6
		HelY	UV + V*	**79.2**	74.6	83.3	**61.3**	55.9	66.4	**39.0**	33.9	44.4
			UV + B	76.4	71.6	80.7	45.3	40.0	50.7	23.9	19.6	28.8
			V + B	73.2	68.3	77.8	49.9	44.5	55.2	26.5	22.0	31.4
			*Dryas**	76.6	71.9	81.0	53.8	48.5	59.2	30.5	25.7	35.6
Bright	Forest	Non-HelY	UV+V	**89.3**	84.9	92.9	**65.6**	59.4	71.5	**45.8**	39.6	52.2
			UV+B	80.2	74.8	85.0	51.4	45.0	57.7	30.0	24.5	36.1
			V+B	75.1	69.3	80.3	53.4	47.0	59.6	31.2	25.6	37.3
			Dryas	78.3	72.7	83.2	54.5	48.2	60.8	32.0	26.3	38.2
		HelY	UV + V	**89.7**	86.1	92.7	**78.1**	73.4	82.3	**66.1**	60.9	71.0
			UV + B	84.3	80.1	88.0	68.9	63.8	73.8	51.9	46.5	57.2
			V + B	83.8	79.5	87.5	68.7	63.5	73.5	55.6	50.2	60.8
			Dryas	86.3	82.3	89.7	72.1	67.1	76.7	60.1	54.8	65.3
	Open	Non-HelY	UV + V	**89.7**	85.3	93.2	**74.3**	68.5	79.6	**50.2**	43.9	56.5
			UV + B	84.2	79.1	88.5	57.7	51.4	63.9	37.2	31.2	43.4
			V + B	78.7	73.1	83.5	60.1	53.8	66.2	36.4	30.4	42.6
			Dryas	81.4	76.1	86.0	59.7	53.4	65.8	37.5	31.6	43.8
		HelY	UV + V	**91.5**	88.0	94.2	**79.5**	74.9	83.6	**70.1**	65.0	74.8
			UV + B	86.6	82.6	90.0	73.2	68.3	77.8	57.0	51.6	62.2
			V + B	85.5	81.3	89.0	72.6	67.7	77.3	60.7	55.4	65.8
			Dryas	87.5	83.5	90.7	76.1	71.3	80.4	63.2	58.0	68.3
Dim	Forest	HelY/non-HelY	UV + V†	76.0	72.5	79.3	**38.0**	34.2	42.0	**16.4**	13.6	19.6
			UV + B	72.6	68.9	76.1	27.9	24.4	31.6	11.3	8.9	14.0
			V + B	**81.5**	78.2	84.5	31.9	28.2	35.7	10.3	8.0	13.0
			Dryas†	78.7	75.3	81.9	32.0	28.4	35.9	11.4	9.0	14.2
	Open		UV + V†	**90.8**	88.3	93.0	**57.8**	53.8	61.7	**33.7**	29.9	37.5
			UV + B	83.6	80.4	86.4	46.1	42.1	50.1	23.7	20.4	27.2
			V + B	89.5	86.9	91.8	48.6	44.6	52.6	21.6	18.4	25.0
			Dryas†	85.5	82.5	88.2	49.6	45.6	53.6	23.2	19.9	26.7
Bright	Forest		UV + V†	**98.7**	97.5	99.4	**86.3**	83.4	88.9	**68.0**	64.1	71.6
			UV + B	93.2	91.0	95.1	75.4	71.8	78.7	54.6	50.6	58.6
			V + B	97.6	96.1	98.6	80.2	76.8	83.3	54.3	50.3	58.2
			Dryas†	97.1	95.5	98.3	78.6	75.1	81.8	56.0	52.0	60.0
	Open		UV + V†	**99.2**	98.1	99.7	**91.3**	88.8	93.4	**75.2**	71.6	78.6
			UV + B	93.9	91.7	95.6	79.4	76.0	82.5	61.0	57.1	64.9
			V + B	97.7	96.3	98.8	83.6	80.4	86.4	60.7	56.7	64.6
			Dryas†	96.8	95.1	98.0	79.9	76.5	83.0	62.8	58.9	66.6

Bold indicates highest percentage in each category.

*Data graphed in Fig. 4.

†Data graphed in Fig. S6.

*The photoreceptors found together in an ommatidium of the butterfly compound eye share a single lens and sample the same part of visual space. For *Heliconius*, the three subtypes of possible trichromatic ommatidia with the following photoreceptor ratios were modeled: 1 UV + 1 V + 6 LW, 1 UV + 1 B + 6 LW, and 1 V + 1 B + 6 LW. For *Dryas*, one type of trichromatic ommatidium was modeled as: 1 UV + 1 B + 6 LW. The relative numbers of photoreceptors are based on available in situ hybridization data for *Heliconius erato*, which found *UVRh* or *BRh* expressed in the R1 and R2 cells and *LWRh* expressed in the R3–R8 cells. No comparable data exist for *Dryas.* We note that actual photoreceptor ratios used in specific tasks such as species recognition are unknown. For *Heliconius*, UV = 355 nm, V = 398 nm, B = 470 nm, and LW = 555 nm rhodopsins; for *Dryas*, UV = 385 nm, B = 470 nm, and LW = 555 nm rhodopsins. Dim corresponds to T = 500; bright corresponds to T = 10,000. See *SI Text* for details of model calculations and Fig. S5 for irradiance spectra. CI corresponds to 95% confidence intervals as calculated using a binomial distribution.

Other Supporting Information Files

Fig. S1 (PDF)

A computational analysis of spectral sensitivity determined by intracellular and electroretinogram recordings from photoreceptor cells in the compound eye of *Heliconius numata*. Black squares indicate original intracellular measurements from (1). Heavy lines show fits to a computational model, a linear combination of R355, R398, R465 and R550. (*A*) Red-dominated recording, SD = 0.041. (*B*) Blue-dominated recording, SD = 0.027. (*C*) UV-dominated recording fit with only R355, R398 and R465, SD = 0.023. Thin lines show fits under the constraint of only a single UV receptor, positioned for best fit. SD are 0.052, 0.047 and 0.101 for panels *A*, *B* and *C*, respectively. The data are best fit by two UV receptors. (*D*) Black squares indicate original electroretinogram measurements from (2). Heavy lines shows a fit to a linear combination of R355, R398, R465 and R550 (SD = 0.056). The thin line shows a fit under the constraint of a single UV receptor, positioned for best fit (SD = 0.080). The data are best fit by two UV receptors.

1. Struwe G (1972) Spectral sensitivity of single photoreceptors in the compound eye of a tropical butterfly (*Heliconius numata*). *J Comp Physiol* 79:197–201.

2. Struwe G (1972) Spectral sensitivity of the compound eye in butterflies (*Heliconius*). *J Comp Physiol* 79:191–196.

Fig. S2 (PDF)

Average reflectance spectra for Heliconiinae wing pigments. Wing colors that are coded as 1 in the Mesquite analysis under various criteria for quantifying variability in the 310-390 nm range are indicated by (*, slope >|±0.2| and max reflectance>20%; †, slope >|±0.1| and max reflectance>20%; ‡slope >|±0.1| and max reflectance >15%).

Fig. S3 (PDF)

Absorbance spectrum of extracted yellow wing pigments subsequently used in mass spectrometric analysis. 3-OHK corresponds to our chemically pure control standard (Sigma-Aldrich, St. Louis, MO).

Fig. S4 (PDF)

Mass spectrometric analysis of yellow wing pigments extracted from *Eueides* and *Heliconius*. (*A*) Chemically pure yellow pigment 3-OHK has a molecular weight of 224.2, producing an expected mass-to-charge ratio (m / z) of 225.1 for the protonated molecule. Pigments extracted from the yellow hindwing bars of *H. melpomene* (*B*) and *H. erato* (*C*) show single major peaks identical to that of 3-OHK, confirming that 3-OHK is the yellow pigment in these species. In contrast to *Heliconius*, yellow pigment extracts from *E. surdus* (*D*), *E. thales* (*E*) and *E. heliconioides* (*F*) show no significant peak at 225.1 (*red arrows*), demonstrating that the yellow pigment in these species is not 3-OHK. The molecular masses observed in these *Eueides* samples do not obviously correspond to any previously described ommochrome pigments. It is notable the *E. surdus* and *E. thales* share most of their major peaks, while *E. heliconioides* has a much different mass spectrometric signature.

Fig. S5 (PDF)

Normalized irradiance spectra of heliconiine habitats measured in Oaxaca, Mexico when heliconiines were flying. The open habitat spectrum was measured under sunny conditions and represents light sampled with the probe perpendicular to the ground facing up. The forest shade spectrum was measured under cloudy conditions and represents light sampled with the probe held parallel to the ground in the south-facing direction. The presence of foliage in the forest shade spectrum is evident by the enrichment of green light compared to the open habitat spectrum.

Fig. S6 (PDF)

Percent of pairs of *Heliconius* yellows compared to non-*Heliconius* yellows that differ by threshold units of just noticeable differences (JNDs) as modeled through the eyes of *H. erato* and *D. iulia* under dim (*A*, *B*) and bright (*C* ,*D*) illumination. Results for the *Heliconius* UVRh1, UVRh2 and LWRh receptor combination are compared to the Dryas UVRh, BRh and LWRh receptor combination. Other possible receptor combinations are shown in Table S3. (*A*, *C*) Forest shade and (*B*, *D*) open habitat irradiance spectra measurements used in the calculations were obtained at localities in Oaxaca, Mexico during times of the day when heliconiines were observed to fly. Threshold (ΔS) units of just noticeable differences (JNDs) of 1, 2 and 3 were chosen to account for the difficulty in estimating true noise values in the butterfly. White represents the *Dryas* visual system and black represents the *Heliconius* visual system. N=621 pairs of colors compared/visual system/habitat. For all thresholds, more pairs of yellows differ by 1, 2 or 3 JNDs under the *Heliconius* visual system than the *Dryas* visual system making it very likely that more of these colors can be discriminated by *Heliconius*. Bars indicate 95% confidence intervals.